A Molecular Analysis of Two Related, c-Myb-Binding Proteins; p160 and p67

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SUMMARY

The *c-myb* proto-oncogene plays a central role in vertebrate haemopoiesis and encodes a DNA-binding transcription factor (c-Myb) that functions to maintain proliferation of immature haemopoietic cells. Both c-Myb and oncogenically activated forms, for example, the viral Myb proteins encoded by the AMV and E26 avian acute leukaemia viruses, are able to directly bind DNA in a sequence-specific manner and transcriptionally activate 'target' genes harbouring their cognate recognition sequence. The DNA-binding and transactivation activities of Myb are necessary for the ability to transform haemopoietic cells.

The activities of c-Myb are regulated in a number of ways, encompassing transcriptional and post-translational mechanisms. The negative regulatory domain (NRD) of c-Myb, located in the carboxyl-terminal region, is implicated as functionally important region required for the down-regulation of c-Myb activity. One identified component of the NRD is a leucine zipper motif, a structure known to mediate protein-protein interactions. Therefore, proteins that interact with the c-Myb leucine zipper may represent crucial regulators of c-Myb activity.

Two related proteins, termed p160 and p67, have been detected within nuclear extracts of murine cells on the basis of their ability to bind to a GST fusion protein containing the c-Myb leucine zipper motif, but not to similar fusion proteins containing mutated leucine zipper motifs (Favier and Gonda, 1994). These proteins are related both structurally and functionally, but possess distinct properties, namely their ability to interact with the c-Jun basic-leucine zipper (bZIP) region, and their distribution amongst the murine cell lines examined.

A partial murine cDNA clone had been isolated using sequence information derived from tryptic peptides generated from purified p67. This thesis describes the complete cloning of cDNA encoding both p160 and p67, and characterisation of these proteins, including the determination of their relationship.

A full-length murine cDNA clone (4.1 kb) corresponding to p160 mRNA was obtained. Database searches indicate that the p160 sequence is novel, and in addition, contains all five of the tryptic peptide sequences derived from p67. Interestingly, the p67-derived sequences are clustered in the amino-terminal region of p160. Northern analysis with p160 cDNA sequences revealed one, ubiquitously expressed mRNA species of 4.5 kb amongst the murine cell lines examined. Based on several lines of evidence, it was hypothesised that p160 and p67 have a precursor-product relationship, whereby p67 is derived from the amino-terminal region of p160 by proteolytic cleavage. In order to further study the properties of p67, a carboxyl-truncated p160 clone (termed p67*) was generated, and used to approximate the properties of endogenous p67.

The authenticity of the p160 cDNA clone was demonstrated by comparative peptide mapping of endogenous p160 and p67 with *in vitro* translated p160 and p67*. An investigation of the binding properties of p160 and p67*, using proteins synthesised *in vitro* and *in vivo*, revealed the same binding specificities as endogenous p160 and p67. The ability of p160 and p67* to interact with wild type c-Myb within the intracellular environment was examined by coimmunoprecipitation of ectopically expressed proteins.

Polyclonal antisera were raised against the amino- and carboxyl-terminal regions of p160. Both endogenous p160 and p67 were detected with antiserum raised against the amino-terminal region, whereas p160, but not p67, was detected with antiserum raised against the carboxyl-terminal region. Detection of endogenous p160 and p67 with the p160 antisera revealed the same subcellular localisation and distribution of these proteins among murine cell lines as previously described binding data.

The subcellular localisation of p67* prompted a further investigation of this protein. Unlike endogenous p67, which was detected within nuclear extracts, p67* was detected only within cytoplasmic extracts. Additionally, p67* lacks carboxyl-terminal residues present within p67. Two approaches were undertaken in order to target p67* to the nucleus; (1) The carboxyl terminus of p67* was extended by generating two additional p160 carboxyl-truncated proteins. (2) The SV40 large T-antigen nuclear localisation signal was incorporated into the carboxyl terminus of p67*. Analysis of the subcellular localisation of the p160 carboxyl-truncated proteins and nuclear-targeted p67* fusion proteins has

revealed potentially important sites/domains within p160 that contribute to transport between subcellular compartments.

The structural relatedness of p160 and p67 was established on the basis of common peptides, as shown by peptide mapping (Favier and Gonda, 1994). Examination of the p160 cDNA sequence and detection of a single mRNA species suggested a precursor-product relationship. This possible relationship was investigated by pulse-chase analyses, and proteolysis assays using total cell extracts and *in vitro* translated p160. Proteolytic cleavage of p160 was directly demonstrated by proteolysis assays. These assays showed that cells in which p67 was detected, contain a proteolytic activity that was able to cleave *in vitro* translated p160 and rapidly generate four major cleavage products, including one of 67 kDa that maps to the amino-terminal region of the p160 precursor protein, in which the p67-derived peptide sequences are located. Furthermore, this proteolytic activity can be specifically inhibited, and is composed of a serine protease(s). Pulse-chase analysis of p160 and preparation of nuclear extracts (from cells in which both p160 and p67 were detected) in the presence of this specific protease inhibitor, indicated that p67 can be generated during the nuclear extraction procedure. Subsequently, preliminary investigations were undertaken to examine whether p67 could be generated *in vivo*.